Exhibit Previously Submitted
U.S. Patent Application Serial No.
08/976,560

Atty Dkt. No.: UCAL142CON
USSN: 08/976,560
Exhibit 1

CERTIFICATE OF MAILING

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Typed or Printed Name	*Cindy Hoang*		
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**DECLARATION OF
L. ALISON MCINNES
UNDER 37 C.F.R. § 1.132**

Address to:
Assistant Commissioner for Patents
Washington, D.C. 20231

Attorney Docket Confirmation No.	UCAL142CON 2046
First Named Inventor	N.B. Freimer
Application Number	08/976,560
Filing Date	November 24. 1997
Group Art Unit	1655
Examiner Name	L. Arthur
Title	*Methods for treating bipolar mood disorder associated with markers on chromosome 18p*

Dear Sir:

1. I, L. Alison McInnes, declare and say I am a co-inventor of the claims of the above-identified patent application. I directed others and personally performed the research leading to the invention disclosed and claimed therein.

2. I have read the Office Action dated April 24, 2001 in this application and understand that the Examiner has rejected pending claims 1-12 and 25-27 on the basis that the specification is not enabling for the full scope of the claims.

3. The data presented below show that, using techniques described in the specification, at least five new polymorphisms, including single nucleotide polymorphisms (SNP), were identified in the narrow interval on chromosome 18p described in the application, which polymorphisms are associated with bipolar mood disorder (BP). Thus, in addition to the polymorphisms already identified in the patent application, and using the guidance provided in the application, several additional polymorphisms were identified that are associated with BP.

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ASSOCIATION OF POLYMORPHISMS WITH BP IN A NARROW INTERVAL ON CHROMOSOME 18P AS IDENTIFIED IN THE INSTANT APPLICATION AND CORROBORATED BY SUBSEQUENT WORK

4.   The instant application provided data showing a positive LOD score for a D18S59 allele with BP in a pedigree analysis; and gave evidence of an association of D18S59 with BP in a population study. The instant application further showed a positive LOD score for a D18S476 allele with BP in a pedigree analysis and gave evidence of association in population studies. In a subsequent study of linkage disequilibrium (LD) on chromosome 18 in a population sample of 69 BP-I patients from the Central Valley of Costa Rica (CVCR), the same D18S59 allele was associated with BP-I. Escamilla et al. (1999) *Am. J. Hum. Genet.* 64:1670-1678; a copy of which is provided herewith as Exhibit 2.

5.   Further genotyping of the 69 affected individuals using four publicly available microsatellite markers delineated a segment of maximal LD with BP-I, covering about 331 Kb. Evaluation of a larger sample (227 patients and relatives, and 26 independent control trios) using these markers showed continuing evidence of LD and haplotype sharing in this sample for this region. Escamilla et al. (2001) *Am. J. Med. Genet.* 105:207-213; a copy of which provided herewith as Exhibit 3.

6.   Thus, the instant application provides evidence of association of at least two polymorphisms associated with BP. This association was corroborated by work published after the filing date of the instant application. These markers are in a narrow interval between SAVA5 and ga203 on chromosome 18p. Within this region, a segment of about 331 kb, and having maximal LD with BP, was further delineated.

AT LEAST FIVE ADDITIONAL POLYMORPHISMS ASSOCIATED WITH BP WERE FOUND IN THE PREVIOUSLY IDENTIFIED NARROW INTERVAL

7.   Using techniques described in the instant application, at least five additional polymorphisms were identified that are located within the narrow interval between SAVA5 and ga203, and that are associated with BP.

8.   As described in detail below, four new microsatellite markers, and 26 new single nucleotide polymorphisms (SNPs) were identified in the narrow interval on chromosome 18p. The results of LD analysis of these 30 new markers, as well as four previously identified microsatellite markers, are displayed in Table 1. Of

the 34 markers presented in Table 1, 16 showed association ($\lambda > 0$) with BP in at least one of the two samples. The p-value for five of these 16 markers was < 0.01. All five of these markers (PH84, PH205, PH202, PH208, and TS30) had estimates of λ near 1.0, indicating that virtually all affected individuals had at least one copy of the associated allele.

METHODS

Sample collection

9.   Two samples were analyzed. In one sample, the patient sample was composed of 227 CVCR BP-I individuals (including the set of 69 patients from Escamilla (2001) that gave the original association evidence in 18p) and their available first degree relatives (total N=563). All affected individuals had at least two psychiatric hospitalizations with the first hospitalization by age 50. A second sample was comprised of these 563 individuals and a set of controls (52 unrelated parents of students recruited from the University of Costa Rica who were selected for CVCR ancestry [at least 5 out of 8 great-grandparents from the CVCR]).

Radiation hybrid and STS-content mapping of markers within the candidate interval

10.   Genetic and physical mapping information was initially obtained from Whitehead Institute for Biomedical Research/MIT Center for Genome Research, Stanford Human Genome Center, GÉNÉTHON Human Genome Research Center, and the Cooperative Human Linkage Center. Radiation hybrid (RH) mapping was used extensively in the early phase of this study to resolve discrepancies in marker order between maps. Specifically, the 83 Stanford G3 radiation hybrid panel was used to map all genetic and STS markers available from public database as well as those developed specifically for the project. In addition to RH mapping, STS-content mapping using BAC (Bacterial Artificial Chromosome) clones from the region of interest was also used routinely to determine the marker order and to complete the BAC contig.

BAC library screening, end sequencing and contig building

11.   Microsatellite and STS markers obtained from public databases were used to screen the human BAC library from Research Genetics (Huntsville, AL) by PCR or to the BAC library from Genome Systems (St. Louis, MO) screen by hybridization according to manufacturers' protocols. BAC DNA from positive clones was prepared, and sequences of the BAC ends were obtained by cycle sequencing the BAC DNA directly with vector primers T7 and SP6, respectively. PCR primers were designed from non-repetitive end sequences and used as STS markers to improve the physical map and the BAC contig construction. The

outlying markers from each side of the contigs were used to screen for overlapping BAC clones to extend the contigs.

Construction of randomly sheared libraries from BACs

12.   BAC DNA was sheared to small fragments of desired size range using a nebulizer. After shearing, the libraries were constructed using established techniques.

Microsatellite and SNP marker development and genotyping

13.   Microsatellite markers were generated by hybridizing oligonucleotide probes for di, tri, and tetranucleotide repeats to randomly sheared sub-libraries made from BAC clones using Quicklite non-isotopic enzyme induced chemiluminescent reagents from Lifecodes Corp. (Stamford CT) following the manufacturer's instructions. Positive clones were sequenced to identify microsatellite sequences and primers were then designed from flanking unique DNA sequence. Primers for amplifying STS markers were also designed using BAC end sequences, and random sequences available within the candidate interval when extensive sequencing of the randomly sheared libraries were done. Primer sequences are publicly available at PNAS Online.

14.   We genotyped the 4 new microsatellites identified by us in sequencing the region. Primer sequences are available on request. Genotyping procedures for the microsatellites were performed using established techniques.

15.   Single nucleotide polymorphisms (SNPs) were identified using SSCP (Single Strand Conformational Polymorphism) analysis of STS markers (all < 300 basepairs in length), using established techniques. We used four unrelated individuals to screen for each SNP. We genotyped the SNPs in patient and control samples using standard SSCP procedures.

Sequencing of the candidate interval and identification of the candidate genes

16.   In the interval of < 3 cM, located within the SAVA5-ga203 interval, randomly sheared libraries prepared from BACs covering this region were sequenced at 10X coverage to discover all sequence information and identify all genes within the interval. More than 10,000 individual sequences from the region were compared by BLAST20 with sequences from publicly available databases and were analyzed using GRAIL21 to identify potential coding sequences. In addition, sequences were assembled using PHRAP 22,

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23, 24 in a single DNA strand of ~331 Kb. The whole sequence was again analyzed using BLAST and GRAIL to aid in gene prediction. These data were displayed in ACEdb (data available from ncbi.nlm.nih.gov) to visualize predicted exons and their relationships to each other.

Statistical analyses

17.   We applied a modified version of Terwilliger's likelihood ratio test of LD to the 4 novel microsatellites and 26 SNPs that spanned our 331 Kb candidate region. For each of these 30 markers we applied this test twice, once in the sample of 227 patients and their available relatives, and also with the addition of the independent controls to the 227 patients and relatives. This likelihood ratio test estimates a single parameter, λ, which quantifies the overrepresentation of an associated marker allele on disease chromosomes versus control chromosomes. λ is related to the common epidemiological parameter of population attributable risk. If the frequency of an associated allele on disease and normal chromosomes is given by p_D and p_N, respectively, then λ is calculated by $(p_D - p_N)/(1 - p_N)$. Only positive associations with disease are permitted, and λ ranges from 0 (under the null of no association) to 1.0 (all disease chromosomes carry the associated allele). Others have shown that λ is the most closely related to the recombination fraction with disease and less influenced by marker allele frequencies than other measures of LD. Because we do not know which chromosome of an affected individual harbors the disease locus, we incorporated a genetic model of disease transmission in the procedure of Terwilliger. Using this model also enabled us to employ data from additional family members other than parents, if they were not available. The same genetic model (mostly dominant with reduced penetrance) was used as in our previous LD papers and in the genome screen of the Costa Rican pedigrees described in McInnes et al. In this model one chromosome of the affected individual is used as a control chromosome. The use of a model is likely to increase the power of the test and the precision of the estimates of λ when the inheritance pattern is approximately known. Using simulated data, Terwilliger shows that his test is conservative.

RESULTS

Marker development and physical map

18.   Based on our previous results (as described in the instant patent application; and in the publications provided herewith) we focused marker development and physical mapping efforts (including direct sequencing) in the <3 cM region between sAVA5 and D18S1231. Within this region we identified 4 new microsatellite markers and 26 SNPs to add to the 4 publicly available microsatellite markers already used

(see Exhibit 3). Based on the extent of haplotype sharing in pedigree CR001 and LD results from the previously used markers, we focused our detailed investigation on the region of about 331 Kb between PH33 and D18S1231 (although in public databases this segment is estimated as being 378 Kb in length, contig NT_011005). Using several sequence analysis tools and database mining procedures (see Methods, above), we determined that this interval contained six known genes (*CENTRIN, CLUL1, TYMS, rTS, YES1,* and *ADCYAP1*, ordered from telomeric to centromeric, with *TYMS* and *rTS* overlapping each other). This order differs in the public database (*CENTRIN, CLUL1, YES1, rTS, TYMS,* and *ADCYAP1,* with no overlap between *rTS* and *TYMS*). All of the genes except "clusterin-like 1 (retinal)" gene [*CLUL1*] have been well characterized previously. *CLUL1* was originally identified during a screen of a human retinal cDNA library for retina-specific genes. The function of this gene is not known; however Northern blot analysis reveals that it is highly expressed in retina with much lower yet detectable expression in several other tissues including brain, kidney and testes.

Genotyping results

19.   We genotyped the 30 new markers in pedigree CR001 and in the CVCR patient and control samples. Results of the LD analysis for these markers (and the four previously available markers reported in ref 8) are displayed in Table 1 (provided herewith as Exhibit 4). Of the 34 markers presented in Table 1, 16 showed association ($\lambda > 0$) with BP-I in at least one of the two samples (that with 227 patients/relatives and that with 227 patients/relatives and the addition of 52 controls). The p-value associated with the estimate of λ was <0.01 for five of these 16 markers, and for four of the five markers the magnitude of association was greater in the sample containing the population controls. All five of these markers (PH84, PH205, PH202, PH208, and TS30), had estimates of λ near 1.0, indicating that virtually all affected individuals had at least one copy of the associated allele. The markers showing LD are clustered in the 19 Kb segment between exon 8 of CLUL1 and exon 1 of TYMS. This segment also contains the minimal region of haplotype sharing within CR001, and for each marker in this segment, the associated alleles seen in the population samples are the same alleles in the shared haplotype in CR001 (last column in Table 1).

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SUMMARY

20.   The data presented herein extend the findings described in the instant patent application. The patent application provided evidence, from both pedigree analyses and population studies, that a number of polymorphisms, including a 154 bp allele of the microsatellite marker D18S59 and a 271 bp allele of the microsatellite marker D18S476, are associated with BP. The patent application described how to identify additional markers, and how to determine whether such markers are associated with BP.
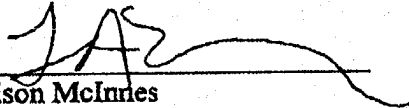
21.   The data presented herein show that, using techniques described in the patent application, several new polymorphisms, located in the previously identified interval and associated with BP, were identified.

CONCLUSION

22. Those in the field, given the guidance in the instant patent application, could identify additional polymorphisms associated with BP.

23. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such will false statements may jeopardize the validity of the application or any patent issuing thereon.

Oct 9 2001
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Date

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L. Alison McInnes

Enclosures: Exhibits 2-4

The American Journal of
Human Genetics

VOLUME 64   NUMBER 6   JUNE 1999

Assessing the Feasibility of Linkage Disequilibrium Methods for Mapping Complex Traits: An Initial Screen for Bipolar Disorder Loci on Chromosome 18

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Summary

Linkage disequilibrium (LD) analysis has been promoted as a method of mapping disease genes, particularly in isolated populations, but has not yet been used for genome-screening studies of complex disorders. We present results of a study to investigate the feasibility of LD methods for genome screening using a sample of individuals affected with severe bipolar mood disorder (BP-I), from an isolated population of the Costa Rican central valley. Forty-eight patients with BP-I were genotyped for markers spaced at ~6-cM intervals across chromosome 18. Chromosome 18 was chosen because a previous genome-screening linkage study of two Costa Rican families had suggested a BP-I locus on this chromosome. Results of the current study suggest that LD methods will be useful for mapping BP-I in a larger sample. The results also support previously reported possible localizations (obtained from a separate collection of patients) of BP-I–susceptibility genes at two distinct sites on this chromosome. Current limitations of LD screening for identifying loci for complex traits are discussed, and recommendations are made for future research with these methods.

Introduction

Identifying genes for disorders with complex inheritance patterns is one of the greatest challenges in biomedical research (Lander and Schork 1994). Such disorders, which include many of the most prevalent human diseases, are difficult to map with standard linkage methods. It has been suggested that the availability of dense marker maps covering the genome will make linkage disequilibrium (LD) analysis a feasible approach for screening the genome to map complex disorders (Risch and Merikangas 1996). Current marker maps are not sufficiently dense to enable such studies to be performed in heterogeneous populations or in populations that were founded in the distant past. However, the success of genome-screening LD-mapping studies of genetically simple and/or rare diseases in recently founded isolated populations (Houwen et al. 1994; Puffenberger et al. 1994; Friedman et al. 1995; Newport et al. 1996) provide the impetus for testing the utility of LD methods for mapping complex diseases in such populations (Escamilla et al. 1996). In populations where randomly sampled patients are on average <20 generations removed from their last common ancestor, LD may be maintained for sizable regions around disease genes. Such LD should be manifested by affected individuals sharing alleles, identical by descent (IBD), at markers spaced at intervals of several centimorgans surrounding

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§Dr. Gallegos died after this paper was accepted for publication. This paper is dedicated to his memory.

a disease gene. We now present the results from the first stage of a study in which LD methods were used to screen for loci that predispose to severe bipolar mood disorder (BP-I), which is common and is almost certainly characterized by a complex mode of inheritance. The study was done in a relatively recently founded isolated population, that of the central valley of Costa Rica (CVCR) (Escamilla et al. 1996), where founder effects have already been observed for several inherited diseases (Saborio 1992; Uhrhammer et al. 1995; Shah et al. 1997).

Despite long-standing evidence that BP-I has a genetic basis (Escamilla et al. 1997), genome scans for linkage have provided equivocal results (Risch and Botstein 1996; Nurnberger et al. 1997) that fail to satisfy the levels of significance suggested for genomewide screens by Lander and Kruglyak (1995). The failure to identify BP-I loci definitively, by standard linkage approaches, probably reflects uncertainty regarding mode of inheritance, high phenocopy rates, difficulty in demarcation of distinct phenotypes, and presumed genetic heterogeneity. LD-based mapping approaches within population isolates may offer a means of diminishing several of these obstacles. An approach (such as LD mapping) that samples individuals from an entire population can more easily ascertain a large set of patients with a narrowly defined, reliably diagnosed phenotype (in this case, BP-I) than linkage-based approaches that require ascertainment of family units with multiple affected cases. Within a population isolate, genetic heterogeneity of BP-I may also be less than in larger, genetically mixed populations, as there is a high probability that individuals with such a phenotype share descent from a few common ancestors.

We collected a sample of patients with BP-I, for LD analysis, by identifying individuals currently living in the CVCR who had known CVCR ancestry. This sample was collected independently of our previous pedigree-based studies of BP-I in Costa Rica. Our aim in the current study was to evaluate the feasibility of identifying BP-I loci by LD screening in this population, as proposed in Escamilla et al. (1996). To do this, we conducted an LD screen of an entire single chromosome (chromosome 18). This chromosome was chosen because previous linkage studies in Costa Rica and in other populations suggested that it possibly contained bipolar disorder loci (Berretini et al. 1994; Stine et al. 1995; Freimer et al. 1996a). Genealogical studies indicated that the individuals in our current study did not share common ancestry over the past several generations (Escamilla et al. 1996). We therefore anticipated that we would not detect random genome regions shared IBD by more than a few individuals and that regions of high IBD sharing would thus be areas containing possible BP-I–susceptibility genes inherited from a common founder.

Samples and Methods

Sample Collection

To diminish the likelihood of investigating phenocopies, we limited the sample to individuals with a definite diagnosis of BP-I, with onset by age 50 years and a history of at least two psychiatric hospitalizations. The 48 patients with BP-I (25 female patients and 23 male patients) in the current study were recruited independently from psychiatric hospitals and clinics in the CVCR. First-degree relatives of patients were also recruited, to determine genetic phase. The study was approved by institutional review boards at the Costa Rican Ministry of Health, the University of Costa Rica, and the University of California at San Francisco, and informed consent was obtained from all participating subjects. Of the 48 BP-I subjects, 8 individuals had both parents available for genotyping, 20 individuals had one parent available, 10 individuals had one or more children available, 1 individual had two siblings available, and 9 individuals had no relatives available. In nuclear families, only one individual (the proband) was designated as affected, and all others were considered to have unknown phenotype. Details of ascertainment and diagnostic procedures, and the clinical and genealogic profiles of the study sample, can be found in Escamilla et al. (1996).

Genotyping

We used 26 markers, spanning chromosome 18, to genotype all 48 affected individuals (as well as 53 relatives, to establish phase). Of the 25 regions, 21 were ≤ 6 cM, and 4 were 6–7 cM. The average distance between markers was 4.8 cM. When choices were available, we chose the most polymorphic marker (Gyapay et al. 1994). The average heterozygosity of the markers used in this screen (in the CEPH pedigree collection) was 0.75. (The only screening markers with heterozygosity values <0.70 were D18S464, D18S60, D18S378, and D18S469.) We screened chromosome 18 at a marker density of 6 cM because available marker maps had gaps ≤ 6 cM, and our goal was to have an equal density of coverage across the chromosome (Gyapay et al. 1994). We chose markers from the maps available, at the time of the study, from Généthon (Gyapay et al. 1994), the Cooperative Human Linkage Center (Murray et al. 1994), and the public database of the Utah Center for Genome Research. Genotyping procedures used for all experiments were as previously described by Di Rienzo et al. (1994). In brief, one of the two primers was labeled radioactively with a polynucleotide kinase, and PCR products were separated, by electrophoresis, onto polyacrylamide gels. Autoradiographs were scored independently by two raters. Data for each marker were entered

into the computer database twice, and the resultant files were compared for discrepancies. Scoring was done without knowledge of affected status.

Simulations

We conducted simulations to evaluate the power of a likelihood-based test of LD (Terwilliger 1995), to detect a result significant at the .05 level, with these assumptions: a 6-cM marker map; a disease gene in the middle of the 6-cM segment; affected subjects, with one copy of the disease gene, separated by 10 generations from a common ancestor; and four equally frequent marker alleles at each marker site. (The disease gene was associated with the "1" allele at the marker locus.) Under these assumptions, and with a phenocopy rate of 0%, normal chromosomes carried each marker allele with a probability of 25% (normal-chromosome distribution), and disease chromosomes carried the "1" allele with a probability of 80%. The probability of disease-chromosome distribution was calculated with the formula $(1 - \theta)^G + [1 - (1 - \theta)^G \times f]$, where θ = recombination fraction, G = number of generations from a common ancestor, and f = the frequency of the allele in the population. Thus, the disease chromosomes carry the "1" allele with a probability of 80% and each of the remaining three alleles with a probability of 6.7%. Because the true genetic structure of bipolar disorder is unknown, we examined several different conditions of etiologic heterogeneity (which would include locus and allelic heterogeneity, as well as phenocopies). We investigated phenocopy rates of 0%, 33%, and 67% (with phenocopy rates of 33% and 67%, the percentages of chromosomes from affecteds with the "1" allele are 62% and 43%, respectively). If an affected individual was randomly selected as a phenocopy (with a probability equal to the phenocopy rate), then the marker allele on all four parental chromosomes was randomly chosen from the normal chromosome distribution. If the affected individual was randomly chosen as a true case, (with a probability of 1 minus the phenocopy rate) the marker allele for one chromosome of that individual was randomly chosen from the normal chromosome distribution, and the other chromosome's marker alleles were randomly chosen from the disease-chromosome distribution. Recombination occurred on parental chromosomes in proportion to the marker map. Marker alleles for nontransmitted chromosomes of the parents were randomly chosen from the normal chromosome distribution. We performed these analyses by using the 48 patients with BP-I plus their available relatives. One hundred replications were performed for each simulation. Available relatives were considered to have unknown disease phenotype. For the 10 affected individuals with at least one child available for genotyping, one chromosome from the affected parent was randomly

simulated to be transmitted to available children, and the other chromosome was randomly selected from the normal chromosome distribution. Although data were simulated for parents of all affected individuals, if parents were not available for genotyping, their simulated genotypes were not used in these analyses.

We also did power simulations (100 replications for each model) of larger sample sizes, using an ideal situation in which both parents are available for genotyping, to aid in planning future studies. In these simulations we used sample sizes of 90, 200, 300, and 400 affected individuals; phenocopy rates of 50% and 75%; and a marker map of 2.5 cM, with all other assumptions as described above. With this denser marker map, at a phenocopy rate of 0%, disease chromosomes carried the "1" allele with a probability of 90%, calculated by the formula $(1 - \theta)^G + [1 - (1 - \theta)^G \times f]$, and each of the remaining three alleles with a probability of 3.3%. Details of the likelihood-ratio test used in analyzing simulation results are described in Analysis.

Analysis

We used two different procedures to identify regions potentially shared IBD by patients with BP-I. The first approach, a search for shared segments, has the advantage of being nonparametric. The second approach, although requiring parameters of the illness to be specified, has the advantage of providing a formal test statistic, allowing for the calculation of P values. These two tests thus offer compensatory strengths and weaknesses when used in the search for genes in a complex disease.

We first searched for shared segments (Houwen et al. 1994). For each individual, we evaluated two marker haplotypes in each of the 25 intermarker intervals, by using a preselected threshold (the possible sharing of a haplotype by ≥50% of patients) to select segments for further investigation. Since this screen does not differentiate between sharing that is IBD and sharing that is identical by state (IBS), use of lower thresholds would lead to too many segments passing the screen.

We also applied a likelihood-ratio test for LD to each of the 26 initially tested markers. This test was done independently of the results of the shared-segment evaluation. We applied a modified version of the procedure of Terwilliger (1995), which only includes case and control chromosomes or chromosomes transmitted and not transmitted to patients. In our sample there were several affected individuals whose parents were not available but whose children were available. DNA from these latter individuals could not be analyzed with the original Terwilliger program but could be analyzed with our implementation of the same procedure, as described by Freimer et al. (1996a). This procedure examines the likelihood that a particular allele (or alleles) is (are) overrepresented on disease chromosomes compared with

Escamilla et al.: LD Mapping of BP-I in Costa Rica

Table 1

Heterozygosity of Markers Used in the Genome Screen of Chromosome 18

Marker Name	Heterozygosity in Généthon Database	Heterozygosity in Costa Rican Sample[a]
D18S1140	.49	.39
D18S59[b]	.81	.81
D18S476[b]	.76	.62
D18S481	.76	.74
D18S391	.75	.69
D18S452	.83	.85
D18S843	NA	.73
D18S464	.65	.51
D18S1153	.78	.69
D18S378	NA	.54
D18S53	.79	.81
D18S453	.82	.81
D18S40	NA	.81
D18S66	.85	.81
D18S56	.73	.74
D18S57	.87	.85
D18S467[b]	.73	.64
D18S460	.62	.67
D18S450	.79	.74
D18S474	.82	.73
D18S69	.79	.78
D18S64	.74	.65
D18S1134	.73	.68
D18S1147	.85	.86
D18S60	.37	.58
D18S55	.77	.80
D18S68	.79	.79
D18S477	.62	.70
D18S61[b]	.87	.86
D18S488	.87	.82
D18S485[b]	.79	.79
D18S541	NA	.63
D18S870[b]	NA	.66
D18S469[b]	.65	.64
D18S874	NA	.64
D18S380	NA	.63
D18S1121[b]	.74	.77
D18S1009	.74	.66
D18S844	NA	.76
D18S554	.82	.79
D18S461	.77	.65
D18S70	.83	.86

NOTE.—NA = data not available.
[a] Allele frequencies were calculated from the entire sample, accounting for known relationships among individuals.
[b] Markers with -2ln(LR) >1.0.

nondisease chromosomes (Terwilliger 1995; Freimer et al. 1996a). A single parameter, λ, is estimated, which quantifies such overrepresentation of marker alleles on disease chromosomes. Designation of chromosomes of probands as disease carrying or non–disease carrying was achieved by specification of a genetic model for the disease. The same model of transmission was used in this LD-likelihood test as was used in the initial genome screen of the Costa Rican families, described in McInnes et al. (1996). In brief, this model assumes that the disease is nearly dominant (assuming penetrance of .81 for heterozygous individuals and .90 for homozygous individuals with the disease mutation), that the population prevalence of BP-I in Costa Rica is .015, and that the frequency of the disease gene in the population is .003. In the likelihood calculations, all possible disease-genotype combinations of all relatives are considered. With the model that was used, in which the disease-gene frequency is very low, the LD-likelihood test, in most cases, treats the probands as effectively heterozygous at the disease locus, and chromosomes of other relatives not occurring in the probands are treated as non–disease-carrying chromosomes. We did not specify a phenocopy rate in the genetic model, because the effect of phenocopies will be absorbed by the parameter λ; the presence of phenocopies in our sample will serve to erode the association between marker alleles and disease and hence will reduce the estimate of λ. Because, in the present LD study, we were attempting to gather further evidence regarding the findings published in our initial genome screen, we limited ourselves to this one model in performing the likelihood analyses. However, both the BP-I family sample and the current LD sample will ultimately be analyzed with use of other models. We considered as promising those markers that gave evidence of overrepresentation of an allele on affected chromosomes, with a $-2ln$(likelihood ratio [LR]) statistic >1.0.

Follow-up genotyping and LD-analysis studies were performed on markers that gave suggestive findings in the shared-segment evaluation. Within each segment that passed the threshold described above, 1–3 additional markers were typed to permit us to test for LD across regions of 1–2 cM. Markers that provided suggestive evidence of LD by the initial likelihood-ratio test, but had not been suggested as promising by the shared-segment screen, were also followed up, in this case by typing two additional nearby markers. In all, a total of 42 markers from chromosome 18 were used to genotype the study sample (table 1 and fig. 1). LD analysis of the additionally typed markers was conducted by use of the likelihood-ratio test.

Results

Simulations

Simulation results for the sample of 48 patients with BP-I and available relatives showed relatively high power to detect suggestions of association ($P \le .05$) with low phenocopy rates (94% for a phenocopy rate of 0%, and 54% for a phenocopy rate of 33%) but a dramatically decreased power under high phenocopy rates (e.g., 9% for a phenocopy rate of 67%). Additional simulations showed that, under higher phenocopy rates, the power to detect LD can be improved by increasing the sample
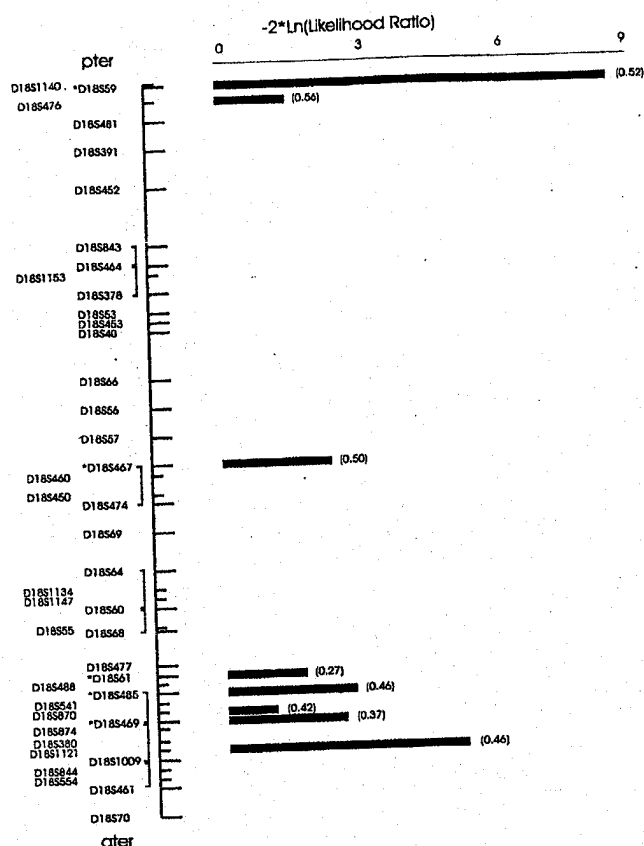
Figure 1   Results from the LD screen of chromosome 18. The 26 markers used in the first stage of the screen are listed in the right column. Sixteen markers used to follow up interesting regions are listed in the left column. Approximate chromosomal locations of the 26 initial markers and the 16 follow-up markers are indicated by long and short tick marks, respectively. The eight segments that passed the initial screen threshold for segment sharing (50% of individuals or 25% of chromosomes sharing a two-marker haplotype) and the five markers that passed the initial threshold for the Terwilliger LR test ($-2\ln$[LR] > 1.0) are indicated by blackened bars and asterisks, respectively. Two marker segments that passed the initial threshold were followed up by at least one marker within the segment, if possible (at the time of the study no markers were available between D18S843 and D18S464, and only one marker was available between D18S464 and D18S378). Markers that passed the initial threshold for the Terwilliger LR test were followed up with two additional markers. These additional markers flanked the original finding. The value of the $-2\ln$(LR) statistic, from the Terwilliger test, is plotted as a solid bar. This statistic is distributed as a one-sided χ^2 random variable with one degree of freedom. The estimate of the λ value, for the eight markers with positive results, is indicated in parentheses after the $-2\ln$(LR) statistic. Markers without a $-2\ln$(LR) statistic plotted had estimates of $\lambda = 0$, with the exception of three markers that had estimates of $0 < \lambda < 0.62$.

size and/or the marker density of screening (table 2). For instance, with a phenocopy rate of 75%, the power increases to 82% with a sample of 300 affected individuals and a 2.5-cM marker map.

Shared-Segment Screen

We evaluated 25 possible shared segments (defined by the 26 markers genotyped in the sample). Eight regions passed the threshold of possible IBD sharing by $\geqslant 50\%$ of patients. These regions were bounded by the following markers: D18S843-D18S464, D18S464-D18S378, D18S467-D18S474, D18S64-D18S60, D18S60-D18S68, D18S485-D18S469, D18S469-D18S1009, and D18S1009-D18S461 (fig. 1).

Linkage-Disequilibrium Testing

Five (D18S59, D18S467, D18S61, D18S485, and D18S469) of the original 26 markers displayed evidence of possible LD, by means of a likelihood procedure ($-2\ln$[LR] statistic >1.0; table 3). Two (D18S59 and D18S61) of these five markers had not been identified as markers of interest by the shared-segment evaluation. D18S59, located near 18pter, displayed the strongest pointwise evidence for LD ($-2\ln$[LR] statistic of 8.3, $P = .002$) of all the markers tested in this sample.

Follow-up of Initial Results

Using the protocol discussed in Samples and Methods, we genotyped additional markers within the segments that passed the shared-segment screen as well as follow-up markers surrounding one (D18S59) of the two markers that had passed only the LD screen. We were unable to follow up one shared-segment region (D18S843-D18S464), because additional polymorphic markers were not available within the segment. We were also unable to follow up the finding for D18S61, for the same reason. Three (D18S476, D18S870, and D18S1121) of the 16 follow-up markers typed displayed additional evidence of possible LD (fig. 1).

These additional results brought to eight the total number of markers with $-2\ln$(LR) statistics >1.0 (table 3). Five of these eight marker loci were clustered within a small region of 18q22-23. The most significant LD in 18q22-23 was observed at D18S1121, with $-2\ln$(LR) of 5.03 and $P = .01$, and two were in 18pter.

For the two 18pter markers (D18S59 and D18S476),

Table 2

Power-of-Likelihood–Analysis Test of LD

	POWER TO DETECT LD FOR SAMPLE SIZE (N) =			
PENOCOPY RATE	90	200	300	400
50%	82%	99%	100%	100%
75%	33%	62%	82%	90%

NOTE.—Assumptions included that subjects were removed from a common ancestor by 10 generations, that a marker map of 2.5 cM was used, and that each marker had four equally frequent alleles. Values are the percentage of replicates to have P values <.05.

Table 3

Frequencies of Marker Alleles Overrepresented in Disease Chromosomes, as Compared with Nondisease Chromosomes, for Markers Where -2ln(LR) >1.0

		FREQUENCY ON	
MARKER	ALLELE	Nondisease Chromosomes	Disease Chromosomes
D18S59[a]	154	.121	.572
D18S476	271	.470	.771
D18S467[a]	172	.384	.693
D18S61[a]	177	.074	.326
D18S485[a]	182	.237	.586
D18S870	179	.405	.657
D18S469[a]	234	.128	.450
D18S1121	168	.171	.553

[a] Markers from the screening stage.

the alleles overrepresented on BP-I chromosomes (154 and 271 bp, respectively) form a haplotype that occurs in 48% of the patients with BP-I. Overall, this haplotype occurs on 26% of the chromosomes of individuals with BP-I and on 4% of the chromosomes not transmitted from parents to individuals with BP-I (definite phase for these two markers could be assigned in 25 patients with BP-I [50 chromosomes] and 25 nontransmitted parental chromosomes). Because the composite genetic and physical maps of the 18q22-23 region had not yet been completed at the time of this study, the relative order of the five markers in 18q22-23, for which evidence of LD was observed, was still too uncertain to permit construction of definitive marker haplotypes in our study sample.

Marker D18S467, in the 18q12.3 region, was the one marker outside 18q22-23 and 18pter to show a -2ln(LR) >1 (-2ln[LR] = 2.5, P = .06). The additional markers used to follow up this result (D18S450, D18S460, and D18S57) displayed no evidence of association.

Marker Heterozygosity in the Costa Rican Sample

We calculated heterozygosity values for the markers used, on the basis of the allele frequencies, estimated from the entire sample, accounting for known relationships among individuals. These heterozygosities are shown in table 1, along with the corresponding heterozygosity values of these markers in the CEPH population, used by Généthon.

Discussion

Screening for Complex Disease Loci by LD Approaches

Our intention in this work was to explore the feasibility of using LD methods to screen the genome for susceptibility genes for a common, genetically complex

disorder. The results obtained in our LD-based search for possible BP-I gene–loci on chromosome 18 were encouraging (specific susceptibility regions were suggested), but they highlight a number of issues that must be considered before LD screening is widely adopted.

Successful application of a shared-segment approach to any LD study depends on (1) a marker-map density that is appropriate to the age of the population isolate being studied and (2) a sharing threshold that will not be too high to allow true IBD areas to be identified and that will not be so low as to include many areas that are IBS false-positive signals. An appropriate marker map for an LD-screening study should have segments of a size expected to be shared IBD by many of the affected individuals. In addition to the density of the marker map used, the number of generations separating affected individuals from their common ancestor and the rate of etiologic heterogeneity in the population will also influence the choice of the sharing threshold, used to trigger further study. For example, if the common (disease–gene bearing) ancestor is removed from the current descendants by >10 generations, the length of true IBD haplotypes shared by ≥50% of the descendants may be <5 cM (and certainly <6 cM, as is the screen used in this study) (Te Meerman et al. 1994; Durham and Feingold 1997). Our choice of a threshold of 50% of affected individuals sharing a possible haplotype therefore effectively meant that we were likely to identify only BP-I genes of a major effect in this population (phenocopy rate approaching zero), and even then, only if the distance from a common ancestor is not > ~10 generations. Although this was probably too stringent a screen threshold, given the complex etiology of bipolar disorder, the alternative we faced—reducing the threshold to a lower percentage of potential IBD sharing—would have drastically decreased the specificity (and hence the utility) of the screen. For instance, in this particular study, lowering the threshold to a possible IBD haplotype shared in ≥25% of the patients would have resulted in 24 of the 25 regions tested being determined as regions of interest. If, in future studies, definite phase information can be set for a greater proportion of the probands (obtained from phasing information supplied by additional relatives) the "possible IBD" threshold will be more useful as a screening criterion at thresholds approaching 25% sharing (almost one in five of the patients with BP-I in the current study had no relative available for phase construction). Finally, regardless of the threshold chosen, there is no widely accepted statistical test available to evaluate the significance of the number of shared haplotypes observed, although several statistical approaches are under development (reviewed by Kruglyak 1997).

The use of markers with low heterozygosity will increase the number of false-positive results in a shared-

segment screen, as some regions may pass the threshold because of IBS sharing of a common allele. For example, the four regions that passed our shared-segment screen, but gave no evidence of LD in the likelihood-ratio tests (D18S843-D18S464, D18S464-D18S378, D18S64-D18S60, and D18S60-D18S68), included markers that had relatively low heterozygosities in the study population (D18S464, D18S60, and D18S378; table 1).

There are two ways to overcome the limitations of shared-segment analysis, as seen in this study. The first is to increase the density of markers in the initial screen (i.e., increase the proportion of BP-I individuals in whom a shared haplotype can be detected, thus decreasing the number of false-negative results). Second, future screening studies may focus on individuals who have available parents (i.e., increase the number of patients for whom we can set phase, thus allowing the threshold to be lowered in a meaningful way and decreasing the number of false-positive results).

For a formal statistical test of LD, we used the likelihood-ratio test rather than another frequently used method, the transmission disequilibrium test (TDT) (Spielman et al. 1993), because data from all 48 patients with BP-I could be used in the likelihood approach. Effective use of the original TDT requires parental genotypes, which were unavailable for 20 of the 48 patients with BP-I. One potential source of false-negative results, in our application of the likelihood-ratio test for LD, is that it is dependent on the specific genetic model for the disease used in the analysis. For instance, the results of the likelihood analysis presented here are applicable only to transmission of dominantly inherited BP-I genes in the CVCR population. The power of the likelihood test is also critically dependent on the polymorphism content of the markers tested and the density of the markers used for a screening analysis.

Evaluation of Potential BP-I Loci on Chromosome 18

Our previous linkage study of BP-I in two Costa Rican pedigrees had provided several possible localizations for BP-I, throughout the genome. Since the 48 Costa Rican patients in the present study (collected independently of the pedigree studies and with no known relation to the pedigree members) are descended from the same ancestral population as the patients in those pedigrees (CR001 and CR004; Freimer et al. 1996b), we had reasoned that LD could be present in the population sample at markers surrounding any true BP-I loci identified in the pedigree study. LD screening of patients with BP-I, in this population, might also yield important BP-I loci that were not identified in the pedigree study. Pedigree-based linkage studies involve selection of certain subsets (individual families) of the population in which there is a clustering of affected individuals. In a complex disease, such

studies may be useful in finding genes of large effect in those particular subsets, but they might not identify loci that are important in understanding the basis of the disease in the general population. In this LD screen of chromosome 18 in Costa Rican patients with BP-I, two regions were highlighted as being of particular interest, and both regions correspond to segments highlighted in the previous pedigree studies from Costa Rica.

We previously highlighted the 18q22-23 chromosomal region (Freimer et al. 1996a) because this area showed the strongest evidence suggestive of linkage in the two pedigrees of any region tested in a genome screen conducted with ~500 microsatellite markers (McInnes et al. 1996). In the pedigree study, portions of a haplotype of >40 cM in this region were shared by 22 of 26 individuals with BP-I (Freimer et al. 1996a), although formal LOD scores for markers in this area were below the level of significance required for proof of linkage. In the current study, five markers in the 18q22-23 region provide possible evidence of LD in Costa Rican patients with BP-I. The marker that gave the strongest evidence of possible LD in the current study, D18S1121, is located within the 3-cM region of highest haplotype sharing observed in the individuals with BP-I from the pedigree study. The specific allele (of 168 bp), which is overrepresented on the disease chromosomes at this locus (D18S1121) in the sample of the population with BP-I, is also the allele that occurs on the putative high-risk haplotype within the pedigrees (Freimer et al. 1996a).

Our pedigree studies had also highlighted a region at 18pter deserving of further study (McInnes et al. 1996), albeit in only one of the two families, CR001. The second-highest LOD score in the genome observed for family CR001 was at D18S59, located near 18pter, and a nearby marker, D18S476, also gave a positive LOD score in this family. This current study of 48 patients with BP-I now provides additional evidence for a BP-I locus in this region, with the same two markers showing evidence of LD. Because genomewide significance levels have yet to be calculated for LD tests (Kruglyak 1997), we can at present only interpret the evidence for LD in the 18pter region (a pointwise P value of .002 for marker D18S59) as being roughly equivalent to Lander and Kruglyak's criteria for suggestive, but not significant, linkage in a genomewide screen (Lander and Kruglyak 1995). The alleles at D18S59 and D18S476 that are overrepresented among the patients with BP-I, from the population sample (154 and 271 bp, respectively), are also overrepresented in the patients with BP-I from pedigree CR001 (all patients with BP-I in family CR001 have at least one copy of the 154 allele at D18S59), possibly indicating that the patients with BP-I in the pedigree share this region IBD with those 48% of patients with BP-I from the population sample who also carry this haplotype.

The third region that showed possible evidence of LD in our population sample was identified through a single marker (D18S467), in the 18q12.3 region. Additional markers typed near this one did not support the initial suggestion of LD in this region. Evidence from a linkage test that yielded a significance level of $P = .06$ would be expected to occur, by chance, ~24 times (about once on most chromosomes) in a genomewide screen.

Our possible BP-I localizations at 18pter and 18q22-23, in the current sample, are distinct from regions on chromosome 18 suggested by other groups as being possibly linked to mood disorder (Berretini et al. 1994; Stine et al. 1995). We detected no evidence of association with these areas (near the centromere and in 18q21) in our BP-I population sample; nevertheless, the power of our current sample is not great enough to rule out these regions as potential BP-I loci in the Costa Rican population. McMahon et al. (1997) have recently reported excess allele sharing in sib pairs at 18S541, which is in the 18q22-23 region, although their affected status included not only BP-I, but also bipolar type II and schizoaffective patients.

Future Directions

The results of this study suggest that shared-segment–screening approaches will only be useful with the development of denser marker maps (Collins et al. 1997) and with the development of tests that permit statistical comparison of disease-chromosome haplotypes with control-chromosome haplotypes. Because the potential advantages of a shared-segment approach are substantial (this type of approach takes maximum advantage of the fact that haplotypes, not just single alleles, are inherited IBD in population isolates, and it is nonparametric), and because marker maps (Dib et al. 1996; Yuan et al. 1997) and statistical methods continue to improve, we remain optimistic about this method of mapping genes for complex disorders.

Both the 18pter and the 18q22-23 regions would have been identified as regions with possible LD at a significance of $P < .05$, even if we had not used the shared-segment approach but had instead screened for evidence by using only the likelihood-ratio test, with the original 26 markers. Our results indicate that, in population isolates, such as the CRCV, and with suitably dense marker coverage, tests similar to the likelihood-ratio test of LD (Terwilliger 1995) are promising tools for genome screening of complex diseases. It is not clear, however, whether currently available tests will be powerful enough to detect unequivocal proof of association in a genomewide scan for such diseases, given sample sizes that are easily obtained. More-powerful tests are needed and may emerge from efforts to develop measures that make use of haplotype information (Service et al. 1999

[in this issue]; Durham and Feingold 1997; Goldin and Chase 1997).

The test of LD screening conducted in the current study points out the need to do a more complete LD study. We thus intend to perform an LD screening analysis. We thus intend to perform an LD screen of chromosome 18, using an expanded sample of patients with BP-I and a denser marker map. The addition of more-polymorphic markers to genome maps, and the application of haplotype-based statistical tests currently under development, should facilitate efforts to definitively identify BP-I susceptibility genes in Costa Rica.

Electronic-Database Information

URLs for data in this article are as follows:

Cooperative Human Linkage Center, http://www.chlc.org/ (for marker maps)
Généthon, http://www.genethon.fr/ (for marker maps)
Utah Center for Genome Research, http://www.genome.utah.edu/ (for marker maps)

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American Journal of Medical Genetics

Founded by John M. Opitz, M.D.

Neuropsychiatric Genetics

The Official Publication of the International Society of Psychiatric Genetics

Genome Screening for Linkage Disequilibrium in a Costa Rican Sample of Patients With Bipolar-I Disorder: A Follow-Up Study on Chromosome 18

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Linkage disequilibrium (LD) methods offer great promise for mapping complex traits, but have thus far been applied sparingly. In this paper we describe an LD mapping study of severe bipolar disorder (BP-I) in the genetically isolated population of the Central Valley of Costa Rica. This study provides the first complete screen of a chromosome for a complex trait using LD mapping and presents the first application of a new LD mapping statistic (ancestral haplotype reconstruction (AHR)) that evaluates haplotype sharing among affected individuals. The results of this chromosome-wide analysis are instructive for genome-wide LD mapping in isolated populations. Furthermore, the analysis continues to support a possible BP-I locus on 18pter, suggested by previous analyses in this population. Evidence for a possible BP-I locus on 18q12.2 is also described. © 2001 Wiley-Liss, Inc.

KEY WORDS: complex phenotype; population isolate; association mapping

INTRODUCTION

There is considerable current interest in the possibility of locating susceptibility genes for complex traits by evaluating linkage disequilibrium (LD) between such traits and densely mapped sets of DNA markers [Risch and Merikangas, 1996]. Several lines of evidence suggest that LD mapping of complex traits may be most successful when performed in samples from genetically isolated populations that have expanded dramatically in size in the relatively recent past [Wright et al., 1999], although some authors have contested this suggestion on theoretical grounds [Terwilliger and Weiss, 1998]. We have focused on using LD approaches to map susceptibility genes for severe bipolar mood disorder (BP-I) in samples of hospitalized patients drawn from the genetically isolated population of the Central Valley of Costa Rica (CVCR). BP-I is an excellent trait in which to test such approaches, as there is strong evidence for its genetic basis [Reus and

Freimer, 1997], yet mapping studies employing standard linkage approaches have provided equivocal results in localizing susceptibility genes for this disorder and no BP-I predisposition genes have yet been identified [Potash and DePaulo, 2000].

In a previous study [Escamilla et al., 1999] we assessed the feasibility of two different LD methods for mapping BP-I, a search for shared segments [Houwen et al., 1994] and a single marker likelihood test [Terwilliger, 1995]. In that study we genotyped a sample of 48 individuals and available relatives drawn from the CVCR using 25 markers spaced at approximately 6-cm intervals along chromosome 18. From simulations reported in that paper, we determined that for reasonable power to detect LD with a single-point likelihood test, a larger sample size and more dense spacing of markers would be necessary than was used in our initial study. We also recognized that detecting LD through the analysis of haplotype sharing in such a sample would require a substantial number of relatives to set the phase for patient chromosomes, as well as a more effective test than a simple comparison of shared segments. In the current study we screened for LD on chromosome 18 at a higher marker density, using a larger sample of BP-I patients (and with more relatives available for genotyping), and employed a newly developed statistical test (termed ancestral haplotype reconstruction (AHR)), designed to find LD via haplotype analysis in samples drawn from population isolates [Service et al., 1999]. This study provides the first implementation of AHR, which serves as a complementary test to the likelihood-based LD analysis method of Terwilliger [1995] (abbreviated here as LD-T). The current screen incorporated a two-phased approach; regions of the chromosome that showed possible LD in an initial phase of genotyping were investigated at higher marker density in a larger sample of patients and relatives. The results of this two-phased chromosome 18 LD screen are reported here.

METHODS

Sample Collection

All subjects were recruited in accordance with the principles of the Declaration of Helsinki and with approval from the Institutional Review Boards of the University of California at San Francisco and the University of Costa Rica (UCR). All probands had a definite diagnosis of BP-I (based on best-estimate diagnostic procedures, as described previously [Freimer et al., 1996a]) with onset by age 50 and a history of at least two psychiatric hospitalizations. Probands were recruited independently from one another from psychiatric hospitals and clinics in the CVCR. First-degree relatives of probands were also recruited where possible to permit determination of genetic phase (for further information on ascertainment and diagnostic procedures, see Escamilla et al. [1996, 1999]). The sample for the phase I screening included 19 probands with two parents available for genotyping, 33 with one parent available, 9 with children available, 3 with children and spouse available, 1 with siblings available, and 4 with no relatives available. The sample for the phase II screening included 62 probands with two parents available, 102 with one parent available, 15 with children available, 7 with children and spouse available, 19 with siblings available, and 22 with no relatives available.

For the LD-T analyses, different weights were given to the genotype results of each proband, based on the degree to which his/her ancestry (at the great-grandparental level) was known to derive from the CVCR. The distribution of CVCR ancestries for probands in the phase I ($N = 69$) and phase II ($N = 227$) samples are shown in Table I. The phase I and phase II samples were equivalent in the proportions of ancestry derived from the CVCR (Table I).

A control sample was recruited from undergraduate students at the UCR. As there are very low tuition costs at the UCR, the largest public university in the country, the student body covers virtually the entire social spectrum of Costa Rica. We sampled 26 students and both their parents (using the latter 52 individuals as the controls, with the phase set from the students' genotypes). Controls had all eight great-grandparents born in the CVCR. Although, given the age of undergraduate students, not all individuals may be past the typical age of onset for BP, we believe they represent a reasonable population sample. They were not sampled with the anticipation of being free of the phenotype studied, but with the expectation that they would be representative of allele frequencies in the general population of the CVCR. As the statistical analyses employed use estimates of population allele frequencies, addition of these control chromosomes provides a more accurate estimate of these frequencies in the population.

Initial Genotyping

For this study we chose 41 microsatellite markers of the highest heterozygosity available to cover chromosome 18 at approximately 3-cm intervals [Broman et al.,

TABLE I. Distribution of CVCR Ancestry for Probands in the Phase I and Phase II Samples

# of GGP from							
CVCR	2	3	4	5	6	7	8
Percent (N) in Phase I sample	0 (0)	1 (1)	12 (8)	4 (3)	16 (11)	7 (5)	59 (41)
Percent (N) in Phase II sample	1 (3)	2 (5)	13 (30)	2 (5)	13 (30)	8 (19)	59 (135)

GGP, great-grandparents.

1998]. The markers were obtained from the Généthon and Cooperative Human Linkage Center (CHLC) sets (see www.genethon.fr and lpg.nci.nih.gov/CHLC, respectively). We genotyped these markers in a sample of 69 BP-I individuals and their available relatives ($N = 162$) and in the control sample. The genotype data were analyzed using the LD-T and the AHR. The results of these analyses are shown in Table II. Genotyping was either semiautomated using an ABI 377 apparatus for markers, for which an assay for this apparatus was already available (22 markers), or by radioactive labeling using a previously described protocol [Bull et al., 1999] (19 markers).

Follow-up Genotyping

Five chromosome segments that included markers that passed a predetermined threshold for further investigation were followed up in a larger sample (phase II) from the CVCR (227 BP-I individuals and their available relatives and CVCR controls). Given the uncertainty regarding the power of LD analysis for such screening, we set a low threshold for considering regions to be potentially interesting (for LD-T, $\lambda > 0.25$ or $P < 0.05$; for AHR, $P < 0.05$). Regions were followed up using additional markers flanking the original marker at a distance of about 1 cm, where such markers were available, otherwise at the nearest available markers. For locations where more than one marker was available, we chose the marker with the highest heterozygosity. These markers were all from the Généthon and CHLC collections, with the exception of sava5 [Vocero-Akbani et al., 1996]. For the phase II markers, genotyping was either automated (2 markers) or manual (16 markers), according to the protocols described above. The markers in each segment were analyzed using both LD-T and AHR.

Analyses

All analyses were performed both with and without utilizing the Costa Rican control sample described above. Addition of the control sample provided more accurate estimation of population allele frequencies. We applied a modified version of the LD-T for association, first proposed by Terwilliger [1995], to each individual marker independently (see Escamilla et al. [1999] and Freimer et al. [1996b] for further details on the modifications to the LD-T). The LD-T assesses the likelihood that a particular allele is overrepresented on disease chromosomes, compared to nondisease chromosomes, and provides a quantitative estimate of this overrepresentation in the form of a single parameter, λ, for each marker. We also applied the AHR test on windows of three markers. AHR compares the distribution of haplotypes in affected individuals with the distribution expected for individuals bearing a disease mutation descended from a common ancestor. Three parameters are estimated under the alternative: the time since a common founder (g), the percentage of chromosomes in affected individuals to have descended from this founder (alpha), and the position of the

disease locus (x). The likelihood was evaluated at five steps (estimates of x) between each marker, at 15 estimates of g, ranging from 10 to 1,000, and at 50 estimates of alpha, ranging from 0.02 to 1.0. While the population history of the CVCR suggests founding in the 16th and 17th centuries, it is possible that disease mutations shared by patients predate the founding of the population. Furthermore, similar distributions for the expected haplotype counts of affected individuals can be obtained at different combinations of g and alpha, indicating differing explanations for the same data. AHR was modified from the form presented in Service et al. [1999] to include linkage disequilibrium between markers under the null, as suggested by McPeek and Strahs [1999]. When a maximum likelihood was found with this range of g, we examined smaller increments of possible g, to further refine the maximum.

Heterogeneity

Heterogeneity in marker allele frequencies between the initial set of probands used in the phase I study and the additional individuals recruited for the phase II study was assessed as follows: Allele frequencies were estimated separately in phase I ($N = 69$ probands) and phase II ($N = 158$ probands), and then in the two sets combined ($N = 227$ probands). Under the null hypothesis of no heterogeneity in allele frequencies between phase I and phase II samples, the difference in the $-2\times$ log likelihood of the combined set and the sum of the $-2\times$ log likelihoods of the separate sets should be distributed as a chi-square random variable with $m - 1$ degrees of freedom, where m is the number of alleles at the marker being tested.

RESULTS

Phase I Screening

In this phase we genotyped 69 affected individuals from the CVCR and 52 control individuals from the same population for 41 microsatellite markers. We assessed LD via the LD-T and by AHR; AHR used only the 55 patients with one or both parents available. The markers that exceeded these thresholds for the LD-T analysis are indicated in Table II; no marker sets exceeded the threshold for AHR.

Phase II Screening

In this phase we followed up the potentially interesting regions from the phase I screen by genotyping an expanded sample of BP-I patients and relatives in any marker that showed a signal in the phase I screen, as well as in two additional flanking markers (at 1 cm distance, if a marker was available; otherwise we typed the nearest available highly polymorphic microsatellite marker). Not all markers were found on one genetic map. Our primary resources were the Marshfield integrated map [Broman et al., 1998] and the Généthon map [Dib et al., 1996]. If a marker could not be found on either of these maps, we attempted to combine

TABLE II. Markers to Exceed the Threshold of $\lambda > 0.25$ or $P < 0.05$ in the Phase I Portion of the Study

	Trios only				Trios plus controls			
Marker	λ	χ^2	P-value	Associated allele	λ	χ^2	P-value	Associated allele
D18S59	0.48	9.18	0.0012	154	0.45	9.28	0.0011	154
D18S1105					0.26	2.55	0.055	101
D18S1163	0.57	2.54	0.055	335	0.43	1.12	0.145	335
D18S467	0.53	4.73	0.014	172	0.48	4.27	0.019	172
D18S469	0.34	2.84	0.046	234	0.26	0.74	0.195	234
D18S1141					0.31	0.95	0.165	275

'Trios only' indicates results from the 69 probands and their available relatives ($N = 162$). 'Trios plus controls' indicates results including the addition of genotypes from the control individuals ($N = 52$).

information from other genetic and physical maps in public databases to make a composite map. Our follow-up areas were in five different regions (Fig. 1). Interval 1 markers were placed on a composite map (sava5-*0.3cM*-D18S59-*0.3cM*-D18S1231-*1.4cM*-D18S1105-*1.5cM*-CHLC.GATA166D05). The markers in interval 2 were all found only on the Marshfield map (D18S967-*4.4cM*-D18S1163-*4.0cM*-D18S843). The markers in interval 3 were found on both the Généthon and Marshfield maps. The total area covered by the three markers in interval 3 was approximately the same in the two maps; however, the intermarker distances were different (Généthon: D18S1157-*1.8cM*-D18S467-*2.3cM*-D18S450; Marshfield: D18S1157-*4.4cM*-D18S467-*0cM*-D18S450). As the differences in intermarker distances between D18S1157 and D18S450 were fairly substantial, we did separate analyses with both maps. Interval 4 was made using a composite map (D18S870-*2.0cM*-D18S469-*0.1cM*-D18S879). All three markers in interval 5 were on both Généthon and Marshfield maps, with nearly the same genetic distances between markers (D18S1122-*1.5cM*-D18S1141-*1.9cM*-D18S70).

The dense marker set was genotyped in a sample of 227 BP-I patients and their relatives (and in a control sample consisting of 52 unrelated individuals from the CVCR). The data were analyzed using both the LD-T and AHR. For AHR, only 164 individuals were used, i.e., those with at least one parent genotyped. For the LD-T, all of the markers in intervals 1, 2, and 4 (sava5, D18S59, D18S1231, D18S1105, 166d05, D18S967, D18S1163, D18S843, D18S870, D18S469, and D18S879) and two of the markers in intervals 3 and 5 (D18S1157 and D18S1122) resulted in an estimate of λ of zero. The LD-T results for the remaining markers in intervals 3 and 5 (D18S467, D18S450, D18S1140, and D18S70) are in Table III. For AHR (Table IV), the strongest evidence of LD in the phase II screen was in regions including the markers that had shown the most evidence for LD in the phase I screen (D18S59 near 18pter and D18S467 in 18q12). The AHR test suggests possible BP-I localizations between D18S59 and D18S1231 (peak lod score of 1.52, equivalent to $P = 0.008$) and between D18S467 and D18S450 (peak lod score of 1.89, equivalent to $P = 0.003$ using Marshfield map distances; peak lod score of 2.03, $P = 0.002$ using Généthon map distances). Additionally, the

alleles associated with BP-I at the markers displaying LD in both the phase I and phase II studies were the same (154 bp at D18S59 and 172 bp at D18S467 and 275 bp at D18S1141), as were the alleles associated with BP-I at the markers displaying LD using both the LD-T and AHR methods in the phase II screen.

11.32
11.31
11.2
11.1
11.1
11.2
12.1
12.2
12.3
21.1
21.2
21.3
22
23

Interval 1 markers

Interval 2 markers

Interval 3 markers

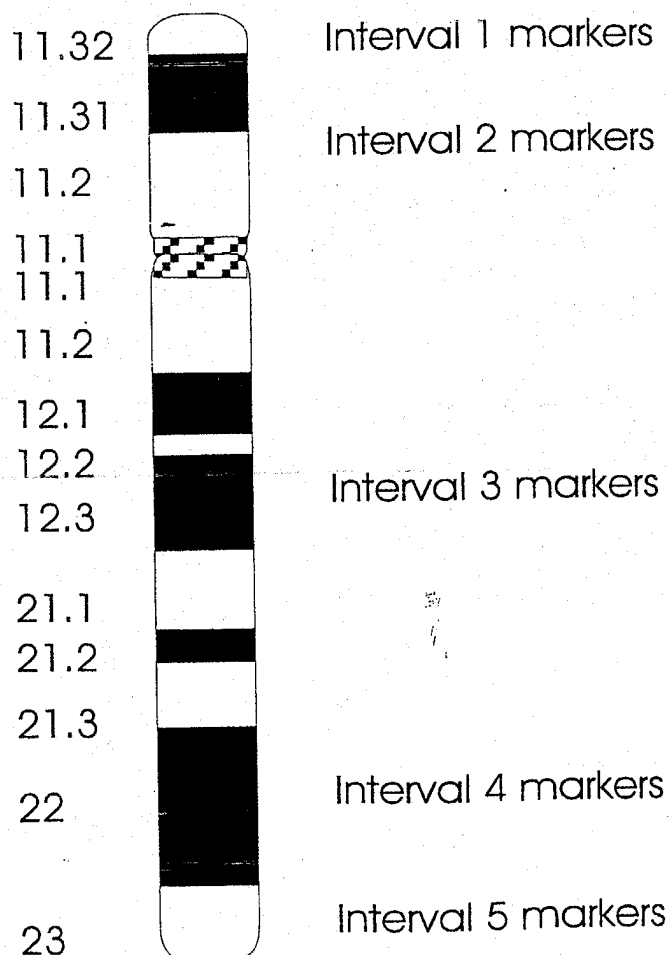Interval 4 markers

Interval 5 markers

Fig. 1. Ideogram of Chromosome 18, showing the five follow-up intervals from Phase II of the study.

TABLE III. Markers in the Phase II Portion of the Study to result in Non-Zero Estimates of λ.

	Trios only				Trios plus controls			
Marker	λ	χ^2	P-value	Associated allele	λ	χ^2	P-value	Associated allele
Interval 3								
D18S467	0.64	5.93	0.007	172	0.61	8.79	0.0015	172
D18S450					0.37	3.40	0.033	204
Interval 5								
D18S1141	0.51	2.07	0.075	275	0.43	1.99	0.079	275
D18S70	0.17	1.77	0.09	114				

'Trios only' indicates results from the 227 probands and their available relatives (N = 563). 'Trios plus controls' indicates results including the addition of genotypes from the control individuals (N = 52).

Heterogeneity Analysis

We performed this analysis to evaluate whether heterogeneity in allele frequencies between sets of subjects sampled over the time course of this study could explain varying results in the different LD-T analyses, in particular at marker D18S59 (between the set of 69 individuals in phase I of the current study and the 227 individuals in phase II of the current study). The heterogeneity method applied here tested for allele frequency differences in the first set of 69 individuals and the second set of 158 individuals. This test failed to reject the null hypothesis of homogeneity considering all markers in the phase II study in a combined test.

DISCUSSION

In this study we have extended our previous efforts to develop LD-based approaches for genome screening to map susceptibility genes for complex traits [Escamilla et al., 1999]; as in our previous study, we used the entire chromosome 18 as a test case. In particular, we applied two different forms of LD analyses to a data set of BP-I patients and relatives, using for the first time a recently developed approach (AHR) based on recon-

structing ancestral haplotypes in isolated populations [Service et al., 1999]. Two of the regions suggestive of LD with BP-I in our previous study (at 18pter and 18q12.2-12.3), using a smaller sample [Escamilla et al., 1999], continue to suggest LD in the present study, using more densely spaced markers and a larger sample size, while a third previously interesting region (18q22-23) shows negligible evidence of LD in the current total sample.

Evidence for LD in the 18pter region (surrounding D18S59) was observed at all stages of our prior and current studies. However, the level of support for LD in this region varied between the initial and subsequent samples and with the type of test used. In our prior study and in phase I of the current study, LD was detected using LD-T but not with haplotype approaches (shared segment evaluation and AHR, respectively), while in phase II of the current study, LD was detected with AHR but not with LD-T. This difference does not reflect heterogeneity between the sample sets, at least as indicated by a formal test for heterogeneity with the markers we have examined. Additionally, regardless of the particular test showing evidence for LD, the same allele at D18S59 has been associated with the disease

TABLE IV. AHR Results From Three Marker Intervals From the Phase II Portion of the Study

3 marker interval and genetic distances	Peak lod score	Location of peak score (recombination fraction)	Estimate of g	Estimate of α	Associated haplotype
sava5-0.3cM-D18S59-0.3cM-D18S1231	0.89	0.0018 from 18S59	298	12%	235-154-10
With controls	0.95	0.0012 from 18S59	296	12%	235-154-10
D18S59-0.3cM-D18S1231-1.4cM-D18S1105	1.33	At 18S59	71	8%	154-10-85
With controls	1.52	0.0019 from 18S59	96	10%	154-10-85
D18S1231-1.4cM-D18S1105-1.5cM-166D05	0.33	0.0118 from 18S1105	6	2%	20-85-308
With controls	0.37	0.0118 from 18S1105	5	2%	20-85-308
D18S967-4.4cM-D18S1163-4.0cM-D18S843	0.41	At 18S967	13	10%	220-202-178
With controls	0.63	0.0087 from 18S967	14	14%	220-202-178
Généthon					
D18S1157-1.8cM-D18S467-2.3cM-D18S450	0.53	0.009 from 18S467	82	28%	128-172-204
With controls	2.04	0.009 from 18S467	92	42%	128-172-204
Marshfield					
D18S1157-4.4cM-D18S467-0.2cM-D18S450	0.19	0.0016 from 18S467	19	6%	128-172-204
With controls	1.85	0.00079 from 18S467	1,000	40%	128-172-204
D18S870-2.0cM-D18S469-0.1cM-D18S879	0.04	0.0079 from 18S870	1,000	12%	179-236-236
With controls	0.18	0.0079 from 18S870	1,000	26%	179-236-234
D18S1122-1.5cM-D18S1141-1.9cM-D18S70	0.24	0.015 from 18S1141	5	2%	136-275-124
With controls	0.19	0.015 from 18S1141	5	2%	136-275-124

'With controls' indicates the AHR results from addition of the 52 control individuals to the sample of 164 probands with at least one parent genotyped.

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phenotype (see Tables II and IV). This associated allele at D18S59 was also observed previously in all BP-I patients from an extended Costa Rican pedigree [Escamilla et al., 1999]. However, the variability in evidence for a disease locus near D18S59 may reflect etiologic heterogeneity in the study sample. Indeed, the AHR tests estimated alpha for D18S59 (the proportion of disease chromosomes descended from a common ancestor) to be as low as 10%.

The evidence for possible LD in the 18q22-23 region has diminished considerably in the current study, compared to earlier work. A linkage study of Costa Rican pedigrees had previously suggested a possible BP-I predisposition locus [Freimer et al., 1996b; McInnes et al., 1996] in the 18q22-23 region; LD screening using 48 BP-I patients from the CVCR had also implicated this region [Escamilla et al., 1999]. While two markers in 18q23 satisfied the criteria in phase I of the current study for further evaluation in phase II, LD-T and AHR P values for all markers tested in 18q23 were clearly nonsignificant in both phases. These results do not negate the linkage and haplotype evidence from the extended Costa Rican pedigrees suggesting a BP-I locus in 18q23, although they may suggest that such a locus does not play a major role in risk for BP-I in this population; further evaluation of BP-I in this region should therefore focus on the extended pedigrees. In both 18p and 18q23, evidence for association has not increased with an increase in sample size. For complex traits with high heterogeneity, it is difficult to predict to what degree a follow-up sample will replicate an original true linkage finding [Suarez et al., 1994]. It is likely that under such a high degree of etiologic heterogeneity, association results such as ours are particularly sensitive to small changes in the composition of a study sample.

In contrast to results on 18q23, evidence for a possible BP-I locus in the 18q12.2-3 region has increased at successive stages of our pedigree and LD studies, and evidence for a possible BP locus in this region has been previously reported by McMahon et al. [1997] in a North American sample (although their definition of affected status was wider than that employed in our study). In our genome screen of the pedigree sample from Costa Rica [McInnes et al., 1996], one of the markers in this interval (D18S450) resulted in a lod score of 1.08 (at a recombination fraction of 0.1) in one of the kindred studied. In our first LD study of 48 BP-I patients from the CVCR [Escamilla et al., 1999], D18S467, from this interval, was associated with BP-I using the LD-T. In phase I of the current study, in which the sample was expanded from Escamilla et al. [1999] by 21 BP-I patients plus additional relatives, the evidence for association at D18S467 increased, with $P = 0.014$. In phase II of the current study, with the sample of BP-I patients expanded to a total of 227, the evidence for LD at this locus increased further ($P = 0.007$ using the LD-T without controls and $P = 0.0015$ with controls). Moreover, one of the markers near D18S467 chosen for phase II of this study also showed evidence of LD (with the LD-T test) at a P value of 0.033 in the total sample. Finally, this region also

shows suggestive evidence of LD using the AHR test in the total sample, at a significance level equivalent to a P value of 0.003 (Marshfield map) or 0.002 (Généthon map).

The overall evidence from the AHR analyses in this region is similar regardless of which map is used; however, the interpretation of the parameters is quite different. When virtually no recombinations are allowed between D18S467 and D18S450, the observed haplotypic diversity in the sample of patients can be explained either by a very ancient founder ($g = 1,000$) or by a more recent founder ($g = 19$), but with few disease chromosomes descended from this founder (alpha $= 6\%$). When more recombination is allowed between D18S467 and D18S450, the estimate of time since a common founder is between the estimates above (average $g = 87$). The interpretations of the parameters are not entirely independent of each other or of the map used in the analyses. Furthermore, the likelihood surface around the maximum likelihood estimates is relatively broad, with other combinations of g and alpha having similar lod scores. The differences in the Marshfield and Généthon maps over this small region are not surprising; it is very difficult to accurately assess genetic distance over regions of less than a few centimeters, given the practical limitations on sample sizes used for constructing genetic maps. Further study is warranted to systematically assess the influence of such map inaccuracies on LD mapping approaches.

This study provides the first use of AHR and a direct comparison of AHR and LD-T. The results of the two tests are not completely comparable, as the sample for AHR testing was smaller than the sample for LD-T testing (as LD-T does not require that all patient chromosomes be phase known). In our previous comparison of these methods by simulation studies [Service et al., 1999], we showed that AHR was more powerful than LD-T in conditions of high etiologic heterogeneity, but that the difference in power between the methods is much less for low heterogeneity or a very old disease mutation (i.e., where it is unlikely that a conserved haplotype could be observed without very densely spaced markers). Our prior simulation comparisons of AHR and LD-T predicted that where alpha is high, both tests will be equally powerful in detecting association, whereas when alpha is low, AHR is potentially much more powerful than LD-T. These predictions may be reflected in our current data. For example, at D18S467, alpha is high for most analyses, and both methods detect an association of similar magnitude. In contrast, at D18S59, alpha is low, and AHR suggests association but LD-T does not. The failure to detect association with AHR in the phase I genotyping study likely reflects low power from the very small sample suitable for AHR testing, as well as the wide spacing of the markers. These comparisons require an important caveat, namely that none of the association results reported here meet unequivocal thresholds for statistical significance; therefore, it is not possible to state, based on this data, how the tests perform in locating a definitive gene predisposition locus for BP-I. Further evaluation of these chromoso-

mal regions with larger samples and additional markers will be required to definitely prove whether BP-I predisposition genes are located at these sites on chromosome 18 and to gain a more clear assessment of the power of the AHR and LD-T approaches for gene mapping of complex traits in population isolates.

ACKNOWLEDGMENTS

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Exhibit 4
08/976,560

Intermarker physical distance	MARKER	227 λ	χ²	p-value	227+ λ	χ²	p-value	CR001 Group A	CR001 Group B	CR001 Group C
unknown	sAVA5*	0.00			0.00					
1058	PH33	0.00			0.66	2.81	0.047			
8977	**Centrin Gene**									
7636	PH49	0.00			0.00					
9850	*CLUL1* **Exon1**									
5231	*CLUL1* **Exon2**									
1340	PH105	0.00			0.00					
3457	PH108	0.00			0.00			X	X	
1230	*CLUL1* **Exon3**									
1224	MPH2 in *CLUL1* Exon 4	0.00			0.00			X	X	
5507	*CLUL1* **Exon5**									
487	*CLUL1* **Exon6**							X	X	
3723	D18S1140*	0.00			0.00			X	X	
1746	*CLUL1* **Exon7**							X	X	X
621	PH77	0.21	0.35	0.278	0.12	0.33	0.284	X	X	X
2473	PH78	0.43	1.04	0.154	0.00			X	X	X
963	CA234†	0.00			0.00			X	X	X
385	*CLUL1* **Exon8**							X	X	X
2464	PH81	0.00			0.00			X	X	X
5447	**PH84**	**0.90**	**10.29**	**0.0007**	**0.78**	**4.40**	**0.018**	X	X	X
1030	*CLUL1* **Exon9**							X	X	X
304	PH89	0.00			0.57	0.85	0.179	X	X	X
1550	**PH205**	**1.00**	**3.98**	**0.023**	**1.00**	**7.14**	**0.004**	X	X	X
494	PH92	0.00			0.59	0.46	0.249	X	X	X
851	*CLUL1* **Exon10**							X	X	X
551	PH211	0.00			0.10			X	X	X
1021	D18S59*	0.35	0.65	0.211	0.00			X	X	X
333	**PH202**	**0.99**	**2.26**	**0.066**	**1.00**	**9.03**	**0.001**	X	X	
1456	**PH208**	**0.96**	**2.20**	**0.069**	**1.00**	**5.96**	**0.007**		X	
586	PH201	0.00			0.00				X	
522	*CLUL1* **Exon 11**								X	
2928	**TS16**	0.00			**0.84**	**4.78**	**0.014**		X	
3756	**TS30**	0.00			**0.88**	**7.31**	**0.003**		X	
15987	**TYMS Exon 1**									
16896	MR16	0.00			0.00					
15312	RTS6	0.00			0.00					
36263	RTS2	0.00			0.00					
602	EXON8	0.46	0.59	0.220	0.00					
35308	EXON7	0.00			0.00					
11345	YES19	0.28	1.37	0.121	0.27	1.89	0.085			
5498	YES12	0.00			0.00					
16614	CA231†	0.00			0.00					
29026	YUM1	0.28	0.28	0.297	0.00					
8161	AT201†	0.42	2.07	0.075	0.32	0.99	0.160			
60719	CA225†	0.00			0.00					
	D18S1231*	0.00			0.00					

Exhibit 4 of 4
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